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**Human Hepatic HepaRG Cells Maintain an Organotypic Phenotype with High Intrinsic CYP450 Activity/Metabolism and Significantly Outperform Standard HepG2/C3A Cells for Pharmaceutical and Therapeutic Applications**

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**Running Title:** Human hepatic HepaRG/ C3A cell comparative study

\*These authors contributed equally as first authors.

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**Abstract:** Conventional *in vitro* human hepatic models for drug testing are based on the use of standard cell lines derived from hepatomas or primary human hepatocytes (PHHs). Limited availability, inter-donor functional variability and early phenotypic alterations of PHHs restrict their use; whilst standard cell lines such as HepG2 lack a substantial and variable set of liver-specific functions such as CYP450 activity. Alternatives include the HepG2-derivative C3A cells selected as a more differentiated and metabolically active hepatic phenotype. Human HepaRG cells are an alternative organotypic co-culture model of hepatocytes and cholangiocytes reported to maintain *in vivo*-like liver-specific functions, including intact Phase 1-3 drug metabolism. In this study, we compared C3A and human HepaRG cells using phenotypic profiling, CYP450 activity and drug metabolism parameters to assess their value as hepatic models for pre-clinical drug testing or therapeutics. Compared with C3As, HepaRG co-cultures, exhibit a more organotypic phenotype, including evidence of hepatic polarity with strong expression of CYP3A4, the major isoform involved in the metabolism of over 60% of marketed drugs. Significantly greater CYP450 activity and expression of CYP1A2, CYP2E1 and CYP3A4 genes in HepaRG cells (comparable with that

of human liver tissue) was demonstrated. Moreover, HepaRG cells also preferentially expressed the hepatic integrin  $\alpha_5\beta_1$  – an important modulator of cell behaviour including growth and survival, differentiation and polarity. Drug metabolite profiling of phenacetin (CYP1A2) and testosterone (CYP3A4) using LC-MS/MS and HPLC, respectively, revealed HepaRGs had more intact (Phase 1-2) metabolism profile. Thus, HepaRG cells significantly outperform C3A cells for potential pharmaceutical and therapeutic applications.

Withdrawal of licensed drugs from the market despite passing both pre-clinical *in vitro* and *in vivo* toxicity testing continues to be a significant problem. Development of improved *in vitro* human hepatic models for pre-clinical drug testing are required to more closely resemble the *in vivo* hepatic environment and minimize inter-species differences<sup>1</sup>. Hence, *in vitro* toxicological models based on human hepatic cell lines would provide more comparable and informative readout than animal models. In order to develop a physiologically-relevant, sustainable and reproducible *in vitro* culture system, the choice of a suitable cell line is critical. Human hepatoma-derived cell lines such as HepG2 and Huh7 are commonly used in early drug safety assessment<sup>2</sup>, but often fail to predict hepatotoxic drugs. The ideal cell model should maintain metabolic pathways such as key CYP450 enzyme activities, possess an intact drug transporter system (hepatic polarity) and be able to offer mechanistic insight into effects of drugs at both the cellular and molecular level. In addition, such culture systems should have a stable metabolic phenotype to further ensure reproducibility and flexibility of use. Primary human hepatocytes (PHHs) are the preferred *in vitro* model for many pharmaceutical and therapeutic approaches. However, limited availability, inter-donor functional and genetic variability and early phenotypic alterations of PHHs cultures restrict their application, such as repeat/ chronic toxicity studies, or availability for cell therapeutics. Integrity of PHHs used for modelling metabolic processes may be in question given the inherent phenotypic

instability of PHHs since upon isolation, the cells are in a state of pre-apoptotic stress with differences in stability of individual CYP450s in culture<sup>3</sup>. This represents a major challenge for pharma where standardization and development of more practical, sustainable, and stable physiologically-relevant alternatives are prerequisite for improving pre-clinical testing outcomes.

Although hepatoblastoma-derived cancer cells, such as the HepG2 cell line are an inexpensive and convenient model, widely used in pre-clinical drug testing, they lack a substantial set of liver-specific functions, particularly, CYP450 activity<sup>4</sup>. The HepG2/C3A cell line (herein designated C3A cells) is a clonal derivative of the HepG2 cell line. C3A cells were selected as a more differentiated and metabolically active hepatic phenotype, compared with the parent HepG2 cell line<sup>5</sup>.

Previously, we demonstrated enhancement of the C3A cell phenotype, including CYP3A4 activity/albumin synthesis, by co-culture with human endothelial cells<sup>5</sup>, and through ‘metabolic’ pre-conditioning<sup>6</sup>, whilst others have used tissue engineering approaches to augment C3A cell metabolism<sup>[7-8]</sup>. Forced transfection of HepG2 hepatic cell lines with CYP2E1-containing plasmids is also performed for highly specific applications, such as ethanol toxicity studies<sup>9</sup>. Notably, C3A and human HepaRG hepatic cell lines have been implemented as the biological component of bioartificial liver systems (BALs)<sup>10</sup>. Suggesting both cell types possess a sufficiently ‘organotypic’ phenotypic and functional properties to support patients with acute liver failure. However, it is not known whether conventional C3A cell monocultures are a practicable model for use in metabolic studies for pre-clinical drug testing or for clinical applications such as BALs<sup>11</sup>. Indeed, limited functionality, low CYP activity, or poor sustainability would represent significant barriers, for BAL application<sup>[12,13]</sup>.

The human HepaRG hepatic cell line has emerged as a potential surrogate to PHHs for pre-clinical hepatotoxicity assays<sup>14</sup>. HepaRGs are a unique (intrinsic), terminally-differentiated co-culture of hepatocyte- and cholangiocyte-like cells containing many functional and phenotypic similarities with PHH<sup>15</sup>. These cells were procured from a young adult female with hepatocarcinoma<sup>16</sup>. HepaRGs are a highly reproducible cell line, without the donor variability seen in PHHs, and as such ensure a consistent and phenotypically stable cell line, presenting a potentially more standardised model<sup>17</sup>. HepaRG cells retain some of the major CYP450 pathways and Phase II, enzymes - as well as production of glucose/glycogen and urea<sup>[15-17]</sup>. These cells also show functional polarity, a hallmark of *in vivo* hepatocyte organisation<sup>15</sup>, with intact Phase III drug transporters. These properties are generally not evident in 'standard' human hepatic cell lines monocultures. Given their potential for pharmaceutical applications and cell therapeutics including BALs, studies comparing such widely used human hepatic cell lines are surprisingly limited, whilst direct comparisons between C3A cells and HepaRG cells have not been previously reported. In this study, we aimed to compare phenotypic and metabolic parameters, including CYP450 activity and metabolism between HepG2/C3A and human HepaRG cells, to assess their value as suitable hepatic models for pre-clinical drug testing and therapeutics.

## **Materials and Methods**

### **Cell Culture**

C3A cells (HepG2/C3A), derivative of HepG2: ATCC® CRL-10741™) were cultured on Corning plates in Minimum Essential Medium Eagle (MEME+; Sigma Aldrich) with 10% foetal bovine serum (FBS, Life Technologies, Paisley, UK) and 1% penicillin/streptomycin (Life Technologies). The culture was kept at 37°C 5% CO<sub>2</sub> until ~80% confluency. HepaRG cells (HRG116 terminally-differentiated cells, BioPredic international, Rennes, France) were

seeded (following the suppliers protocols) on Corning plates and cultured to confluence at 37°C 5% CO<sub>2</sub> for 7 days.

### **Immunocytochemistry**

On culture day 8, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.1% Triton X-100 in Tris Buffered Saline (TBS), then blocked with TBS containing 5% Bovine Serum Albumin (BSA; Sigma-Aldrich) - before incubation with primary (rabbit) anti-human CYP3A4 antibody (AB1254, Chemicon, Millipore, Hertfordshire, UK) (1:800) and TRITC-conjugated Phalloidin (Sigma Aldrich) (1:100). Cells were then treated with appropriate secondary anti-rabbit antibody (Alexa Fluor® 488) prior to (nuclear) staining with DAPI (1:5000) for 5 min. Cell morphology was assessed under phase contrast and for immunocytofluorescent staining using an EVOS AUTO FL microscope (Life Technologies). Images were merged using ImageJ 1.47v (National Institute of Health, Bethesda, MD, USA).

### **Biotransformation potential**

#### **Cytochrome P450 enzyme activity**

Cells were treated for 24 hr with prototypical inducers 50µM omeprazole (CYP1A2) or rifampicin (CYP3A4), with or without addition of CYP450 isoform-specific specific inhibitors 25µM Fluvoxamine (CYP1A2) or Ketoconazole (CYP3A4), in MEME (C3As) or hepatocyte induction medium (HepaRGs). Cells were then washed twice with HBSS. Specific CYP450 enzyme activity was subsequently assessed using a non-lytic luminescence assay using specific kits for CYP1A2 and CYP3A4, following the manufacturer's instructions (P450-Glo, Promega, Southampton, UK). Bioluminescent signals were detected with a GloMax-Multi+ Microplate Multimode Reader (Promega). Individual luminescent assay readings were background-corrected and normalized to cellular ATP content.

## **Phase 1 and 2 metabolism of Phenacetin and Testosterone**

In order to profile relative drug metabolism in each cell type, metabolites formed by CYP1A2 or CYP3A4 hepatic metabolism were assessed following exposure to 50  $\mu$ M phenacetin (CYP1A2), or testosterone (CYP3A4) for 2 hr at 37°C. Reactions were stopped on ice, cell supernatant (medium) samples stored at -80°C before analytical measurements using HPLC (testosterone) or LC-MS/MS (phenacetin).

### **Phenacetin metabolism: Liquid chromatography-mass spectrometry**

Phenacetin metabolism was assessed using an ABI Applied Biosystems 5500 QTrap Mass spectrometer and AB Sciex software, Analyst 1.5.1; and LightSight software to identify metabolites. Methods are described in detail in Supplementary Information.

### **Testosterone**

Relative turnover and metabolic breakdown of testosterone was assessed by HPLC-UV with absorbance at 254nm. Samples were enriched with an internal standard (20  $\mu$ g/ml 11  $\alpha$ -hydroxyprogesterone in methanol) and extracted with dichloromethane before they were resuspended in 30% methanol and injected (25  $\mu$ L) onto an Eclipse XD8-C18 3  $\mu$ m 3 x 100 mm column (Agilent, Cheadle, UK). A series of calibration standards were prepared containing 6 $\beta$ , 7 $\alpha$ , 16 $\alpha$ , 16 $\beta$  and 2 $\alpha$ -hydroxytestosterone, plus internal standard. Androstenedione and testosterone were analysed at the start of each run at 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 nmoles in 30% methanol.

### **Flow Cytometry**

Integrin expression of C3A cells was compared with HepaRG cells, including the progenitor HepaRG101 cell line - and following differentiation to terminally-differentiated HepaRG116 cells. This allows assessment of integrin expression in C3A cells, known to express foetal



markers (eg alpha-fetoprotein), with both the HepaRG101 (hepatoblast-like) progenitors, and their derivatives (HepaRG116), as comparators. To assess expression of integrins, single cell suspensions were enzymatically recovered using xeno-free Tryple (Life Technologies) – which protects cellular surface proteins, washed and resuspended in FACS-PBS (PBS containing 0.1% BSA and 0.1% sodium azide), for simultaneous staining with a panel of fluorochrome-labeled integrin antibodies: CD29-BV510, CD49f-BV451, CD49d-FITC, CD49c-PE, CD49a-APC-Vio770, CD49b-PE-Vio770, CD49e-APC, (all Miltenyi Biotec). Cells were incubated with antibodies at 4°C for 20 min., washed twice and re-suspended in FACS-PBS. Unstained cells were included as controls, whilst dead cells and debris were excluded from the analysis based on scatter characteristics. Data for at least 10,000 live events per sample were acquired using a MACSQuant Analyzer (Miltenyi Biotec) and was analysed using FlowJo version 9.6.7 software (FlowJo LLC). Data are presented as percentage positive staining.

### **Statistical Analysis**

All experiments were performed with three to eight technical replicates from a minimum of three independent biological experiments. Results are presented as mean  $\pm$  standard error of the mean (SEM), and individual groups were (generally) compared with a two-tailed unpaired Student t-test to test significance as indicated. A p-value lower than 0.05 was considered statistically significant.

## Results

### Phenotypic profiling of HepaRG and HepG2/C3A cells

HepaRG cells formed terminally-differentiated *in vivo*-like hepatic cords and cholangiocyte-like cells (fig. 1a) with functional polarity, as evidenced by punctate staining of F-actin bands indicative of bile-canalicular structures [phalloidin-staining, fig. 1c]; these could also be observed in C3A cells (fig. 1d), although with less pronounced staining. In contrast, high CYP3A4 protein expression (fig. 1c; green-staining) with strong expression of CYP2E1, CYP1A2 and CYP3A4 genes, was evident only in HepaRGs; indicative of high metabolic competence for drug metabolism (fig. 1e).

### Biotransformation potential

#### CYP450 enzyme activity and specificity

HepaRG CYP1A2 (fig. 2a) and CYP3A4 (fig. 2c) activity was significantly higher than that measured in HepG2/C3A cells [grey bars;  $p < 0.0001$ ]. In fact, relative luminescence measured in HepG2/C3A cells was at levels of blank controls. Specificity of CYP450 isoform induction was confirmed with the specific inhibitors [chequered grey bars]: Fluvoxamine (CYP1A2) and Ketoconazole (CYP3A4).

#### Phase 1 and 2 metabolism of Phenacetin and Testosterone

To further investigate the extent of CYP1A2 and 3A4 metabolic activity in each cell type, phenacetin (CYP1A2) and testosterone (CYP3A4) metabolism were used to assess metabolic competency. Compound turnover, and ratio of analyte to metabolite was assessed using LC:MS/MS (phenacetin) and HPLC (testosterone). Compared with C3A cells, HepaRG cells showed significantly higher *turnover* of phenacetin ( $28.5 \pm 4.4\%$  versus  $11.7 \pm 0.6\%$ ;  $p < 0.01$ )

and testosterone ( $56.5 \pm 7.6\%$  versus  $2.0 \pm 0.2\%$ ;  $p < 0.001$ , respectively), with a wider spectrum and more refined metabolic breakdown of phenacetin and testosterone in HepaRG cells.

### **Phenacetin**

HepaRG cells yielded a higher turnover of phenacetin and gave a clear yield ratio of analyte to metabolite via end-point analysis (fig. 3a). We were able to characterize de-demethylation of our substrate, phenacetin, by observing significant mono- and tridemethylation of the parent metabolite into paracetamol (fig. 3b-d). In addition, characteristic secondary metabolites of sulfonation and glucuronidation were detected (data not shown). Such broad-spectrum metabolism is not seen in C3A cells, where no secondary metabolites were detected.

### **Testosterone**

Given each compound has its own unique absorbance, the chromatographic elution time of testosterone was compared in *in vitro* samples to that of known calibration standards, and HPLC-UV absorbance was assessed using a photodiode array detector. In contrast with C3As, HepaRG cells metabolised >50% of (total) testosterone – producing a major metabolite (6- $\beta$ -hydroxy-testosterone), as well as a panel of other secondary metabolites (fig. 4a) whilst C3A cells only showed presence of relatively minor metabolites, with low turnover of the parent compound (fig. 4b). Using HPLC we were also able to measure the relative *turnover* of testosterone by hydroxylation (see fig. 3d) cells, while no trace of this metabolite is present in C3A cells.

## Flow cytometric integrin expression in HepaRG and C3A cells

Expression of integrins CD49a-f ( $\alpha$  sub-units 1-6) and CD29 ( $\beta_1$  sub-unit) by C3A, HepaRG101 and HepaRG116 cells is shown in fig. 5. C3A cells expressed CD49a, CD49b, CD49f and CD29 but showed no staining for CD49c-e. The immature/undifferentiated hepatoblast-like cell line HepaRG101, showed a comparable phenotype, expressing CD49a, CD49b, CD49f, although the frequency and level of expression of CD49f was lower than seen for C3A. In addition, HepaRG101 cells expressed CD49e, which was absent from C3A cells. HepaRG116 differentiated cells show hepatocyte, biliary and epithelial morphologically (fig. 5).

## Discussion

Appropriate *in vitro* human models are urgently required to help reduce high drug attrition rates by detecting toxicity earlier and improving predictive outcomes, which may help reduce the reliance on subsequent animal testing in drug development. We compared two candidate human hepatic cell lines, HepaRG and C3A cells and demonstrated differential organotypic properties. Only HepaRG cells retain differentiated morphological, phenotypic and augmented functional features for context-specific applications such as in early-phase drug discovery and development, or BAL use. HepaRG cells may represent a more physiologically-relevant pre-clinical platform for CYP450 activation/ inhibition studies, safety pharmacology, as well as drug-drug interaction studies.

Comparative assessment through characterization and validation of functional and phenotypic properties of candidate human hepatic cells, for a defined operational task, are not widely reported<sup>18</sup>. Compared with C3As, HepaRG co-cultures, exhibit a more organotypic phenotype, including evidence of hepatic polarity with strong expression of CYP3A4 (fig. 1),

the major isoform involved in the metabolism of over 60% of marketed drugs<sup>[12-13]</sup>. Similarly, greater expression of CYP1A2, CYP2E1 and CYP3A4 genes in HepaRG cells, compared with C3As, is comparable with that of human liver biopsies, whilst HepG2 have also been shown to express very low CYP450 levels<sup>16</sup>.

Analytical techniques including LC-MS/MS and flow cytometry allow candidate *in vitro* models to be interrogated for cell type-specific markers and drug reactive metabolite formation – allowing intrinsic (predictive) drug clearance rates to be calculated. Both turnover and the profile of HepaRG phenacetin and testosterone metabolites, is significantly broader compared with C3As (fig. 3-4), suggesting their usefulness in predictive drug testing. Moreover, LC-MS/MS profiling of phenacetin showed that only HepaRG cells catalyzed a range of complex phase 1 (oxidation, mono-demethylation and tri-demethylation) and phase 2 (sulfonation and glucuronidation) reactions (see fig. 3b).

Many *in vitro* drug testing assays use HepG2 cells, whilst the HepG2 derivative<sup>[19-20]</sup>, C3A cells, have been utilized in tissue engineering and cell therapeutic applications, such as the extracorporeal liver assist device (ELAD) system. Although the ELAD system failed to meet expectations in clinical trials<sup>21</sup>. It is well known that detoxification capacity and mixed function oxidase activity is very poor<sup>22</sup>, in HepG2 cells, which require manipulation to stimulate CYP450 activity such as transfection<sup>23</sup>, whilst metabolic preconditioning<sup>6</sup> can enhance functional suitability for BALs, drug safety or pharmacological experiments. Although useful in proliferation assays, further reported disadvantages of HepG2 cells, which we show in the present study, include lack of specific liver function (CYP450 activity/metabolism) that potentially miss significant toxic effects of candidate compounds under investigation.

C3A cells are, however, a useful model for context-specific applications including predictive modelling of hepatotoxins<sup>24</sup>, metabolomics and tissue engineering approaches including 3D-spheroid formation<sup>7</sup> and in microfluidics devices<sup>8</sup>, which improved phenotype and functionality. This is equally true of HepaRG cells which have been utilized in clinical applications such as BALs, hepatitis research and drug-induced liver injury (DILI)<sup>[15-16]</sup>.

Our study supports preferential usage of HepaRG over C3A cells in such applications<sup>[12, 13]</sup>, due to their reproducibility and stability (>28 days) in culture, coupled with a significantly greater array of gene and enzymatic mixed function oxidases (fig. 2). In agreement, other reports show these cells exhibit Phase I-III metabolism<sup>25</sup>. Indeed, Gerets *et al.*<sup>26</sup> compared HepG2, HepaRG and PHHs assessing gene expression and employing real-time impedance biosensing with various CYP450 assays containing prototypical inducers. They found HepaRG cells to be the most inducible model, and metabolically more closely related to PHHs than HepG2. When comparing gene expression of Phase I, II and III mRNA between HepG2, HepaRG and PHH cells, they also found that HepG2 cells expressed the lowest mRNA values for almost every gene tested<sup>22</sup>. Altogether, these studies suggest HepaRG may be a useful surrogate to PHHs.

We performed flow cytometric analysis to assess and compare the expression profile of cell surface integrin receptors. Integrin-mediated cell adhesion sites link the actin cytoskeleton with the extracellular matrix, and are important modulators of cell behaviour including growth and survival, differentiation and polarity<sup>27</sup>. Expression profiling reveals that HepaRG116 differentiated cells show markers associated with hepatocyte, biliary and epithelial morphologically (fig. 5). In contrast with C3A, which was derived from a hepatocellular carcinoma patient, the non-tumourigenic HepaRG cells, notably express the most abundant hepatocyte integrin  $\alpha_5$  (the fibronectin receptor,  $\alpha_5\beta_1$ ), important in

development and cell differentiation processes. Therefore, HepaRG cells may represent a valuable model to study hepatic cell-cell/ cell-matrix interactions.

Given that hepatic clearance of drugs depends on the activity of transport proteins located on the bile canalicular membranes, the unique properties of HepaRG polarity (Fig. 1), may permit focused approaches for cholestatic drug-based DILI studies as well as drug-drug interactions; a significant bottleneck in the drug development pipeline<sup>15</sup>. This, together with the broad hepatic functionality of the HepaRG *in vitro* co-culture system, also offers an opportunity to establish robust ‘disease-in-a-dish’ models and offer insight into the interaction between hepatocytes and cholangiocytes, a main feature of *in vivo* hepatic organisation.

One disadvantage of the HepaRG cells is currently their cost when compared with the widely-available HepG2/C3A cells, which can be propagated through multiple passages. This is perhaps offset to some degree, by the stability of the HepaRG cells for a variety of time-sensitive experiments such as: acute, chronic or repeat-dose hepatotoxicity studies; over 4 weeks, so enabling a larger number of experiments with less variability and more stability.

In conclusion, our comparative study supports human HepaRG cells as a more pertinent organotypic *in vitro* human model, compared with C3A cells, which may permit more predictive pre-clinical screening of drugs and could potentially reduce drug attrition, DILI and animal testing, leading to lower drug development costs. Defining physiological properties of such hepatic models, may inform future human liver tissue modelling strategies for a variety of pharmaceutical and therapeutic applications.

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**Figure 1**

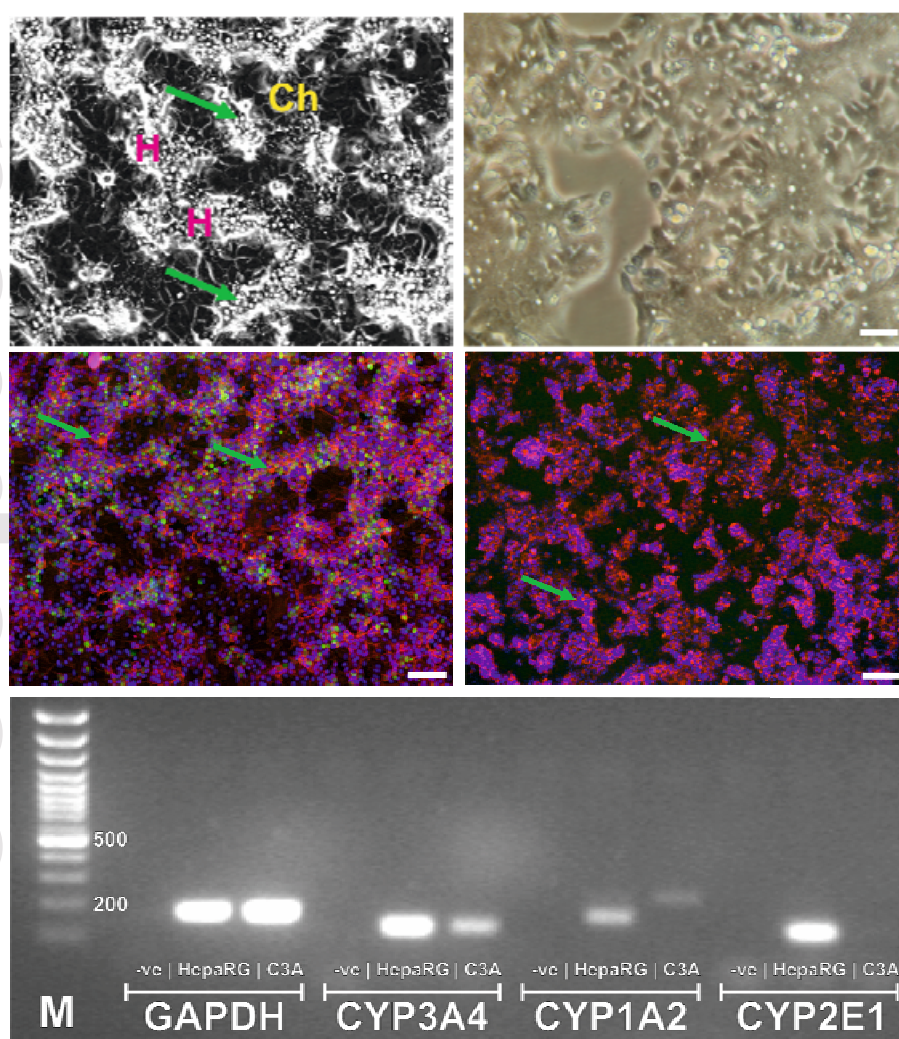


Figure 2

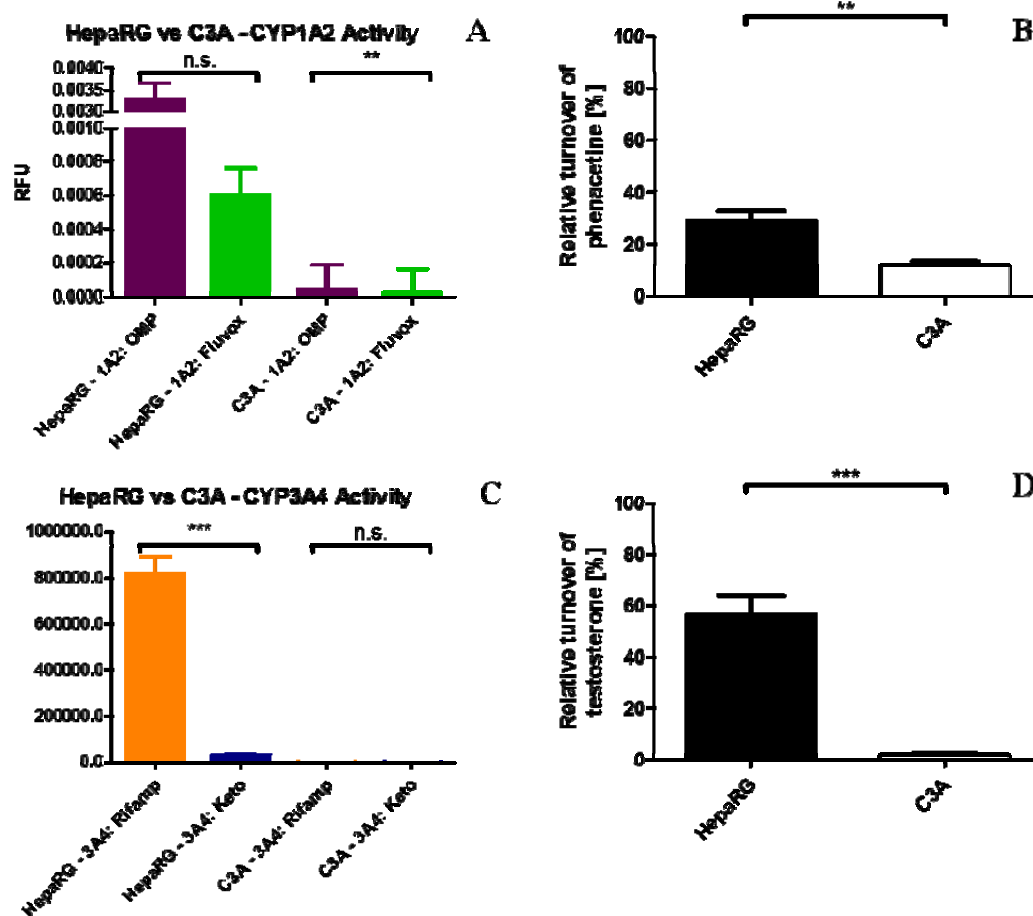


Figure 3

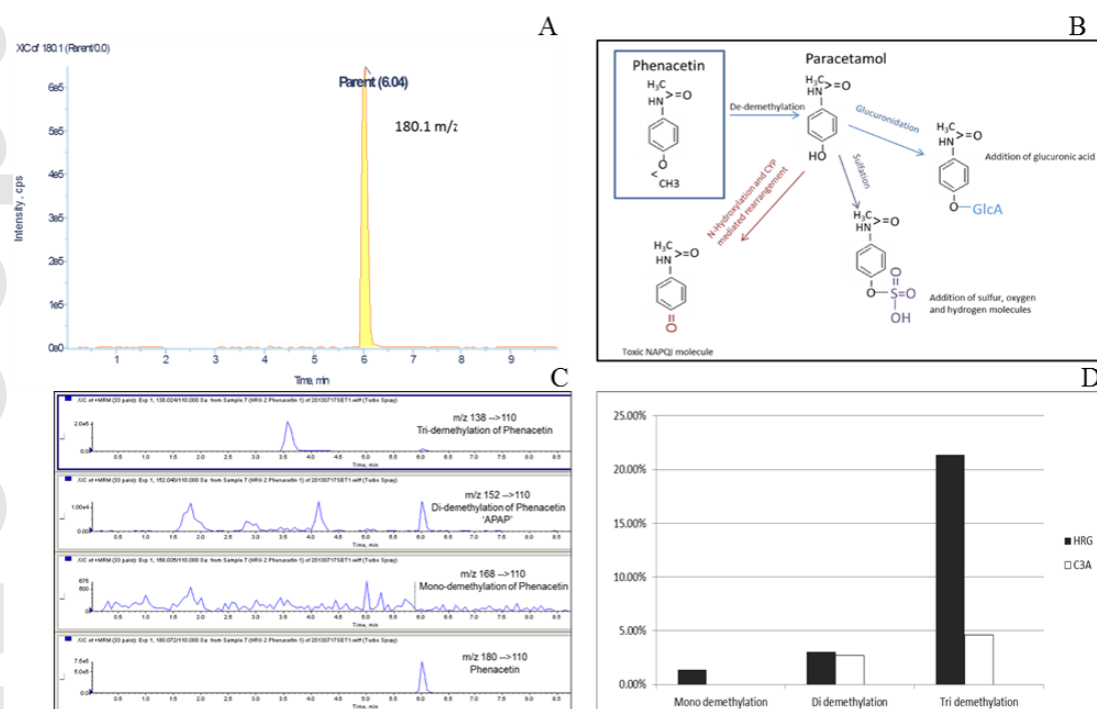


Figure 4

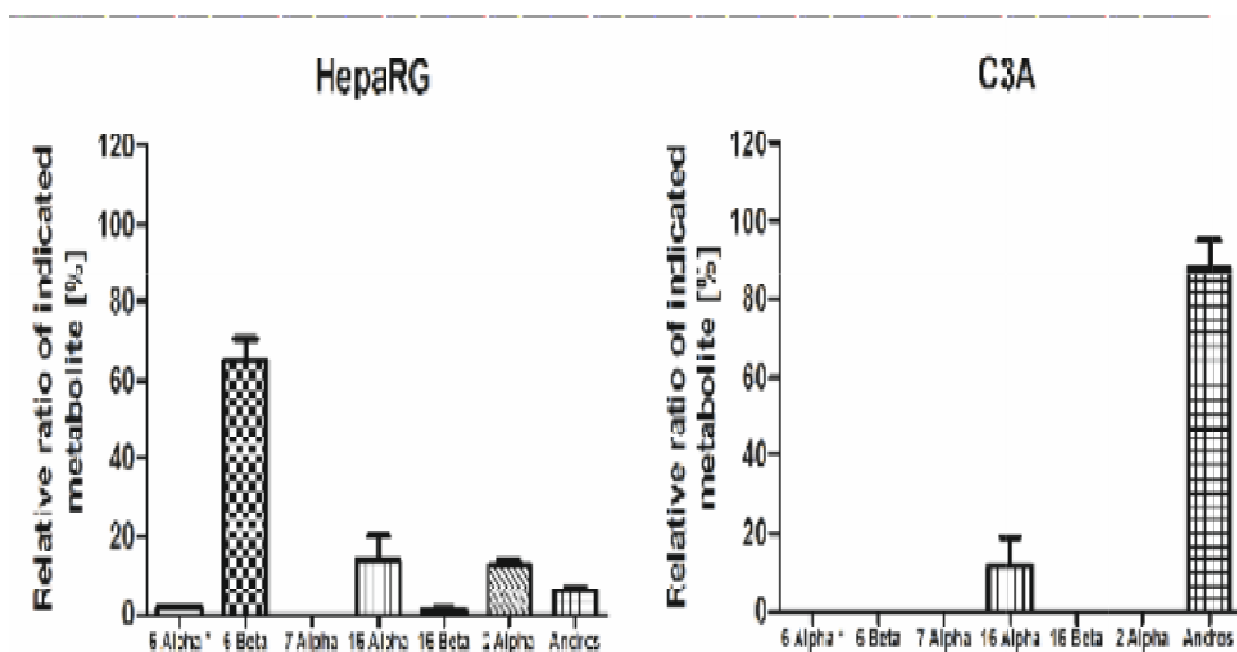
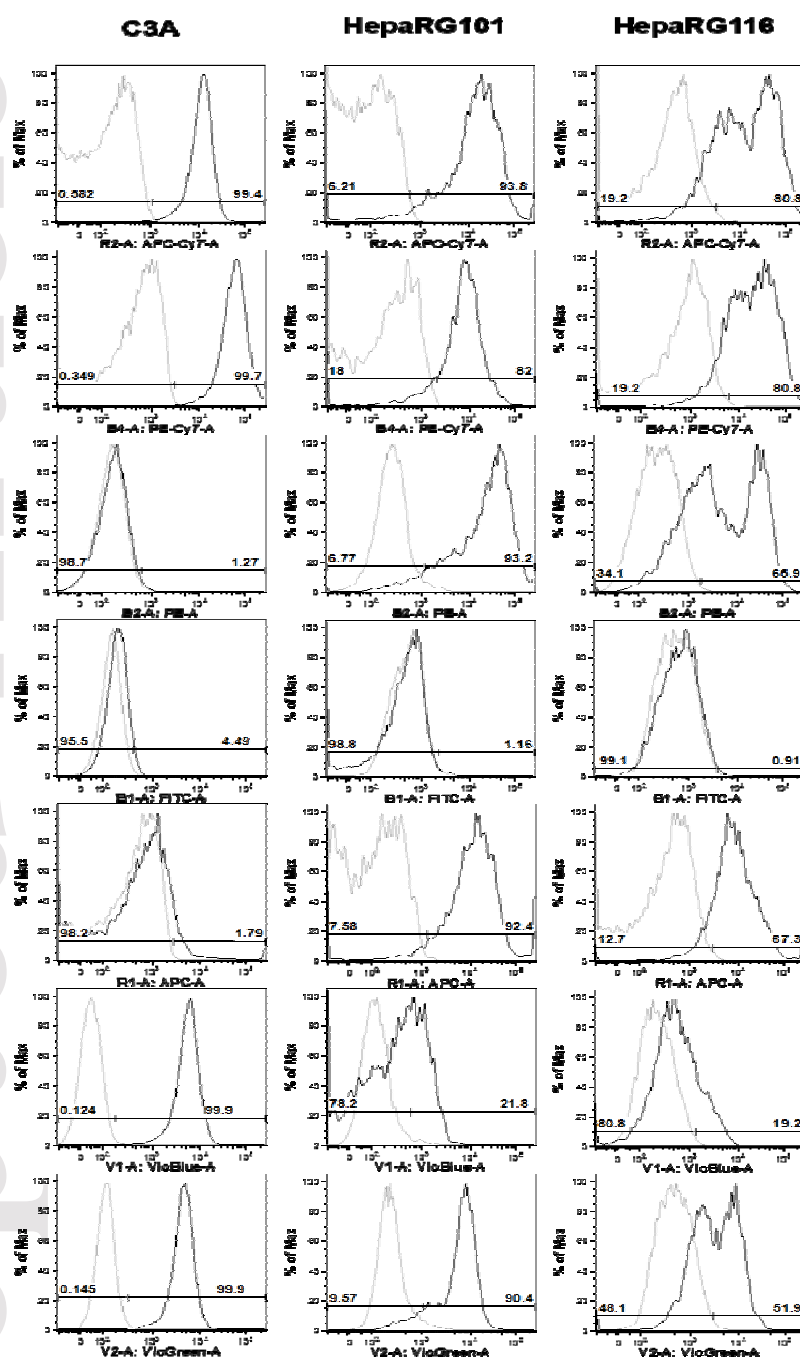


Figure 5



Figures 1-5: Legends

Fig. 1 Phenotypic profiling of human hepatic HepaRG compared with C3A cells

HepaRG cells are an intrinsic co-culture of hepatocyte- and cholangiocytes (biliary-like cells (denoted *Ch*, in A) with islands of hepatocytes (*H*) with sharp refractile borders/round nuclei, and bile canaliculae (arrows) interspersed with a thin layer of cholangiocytes (panel A).

C3As form a confluent monolayer of hepatocyte-like cells, with less prominent bile canaliculi/ refractile borders (B). (C) HepaRG cells exhibit a highly differentiated phenotype (culture day 8), including: Tri-colour fluorescent-staining revealed extensive hepatic CYP3A4 enzyme activity (green); punctate staining of F-actin bands, indicative of bile-canalicular structures [red phalloidin-staining, green arrows]; with *in vivo*-like hepatic cords and cholangiocyte-like cell 'voids'. In contrast, in C3A cells CYP3A4 enzyme activity is undetected (D), with low CYP450 gene expression/metabolic competence in comparison with high CYP2E1, CYP1A2 and CYP3A4 gene expression in HepaRG cells, following end-point PCR (E).

**Fig. 2 Metabolic profiling of human hepatic HepaRG compared with C3A cells.**

Measurement of prototypical inducers Omeprazole (CYP1A2) and Rifampicin (CYP3A4) in both HepaRG and C3A cells (A, C). Fluvoxamine, a highly-specific inhibitor of CYP1A2 and ketoconazole, a highly-specific inhibitor of CYP3A4, were used to demonstrate specificity. Relative metabolic turnover (ie. of total drug added to culture medium) of phenacetin (B) and testosterone (D). Results are presented as mean  $\pm$  SEM, and individual groups were compared with a two-tailed, unpaired Student t-test to test significance as indicated. A p-value  $<0.05$  was considered statistically significant.  $P<0.01^{**}$ ;  $p<0.001^{***}$

**Fig. 3 Representative chromatograph extracted from Light Sight software:**

Peak of parent compound phenacetin at its unique 180.0 m/z ratio (A). Schematic diagram showing Phase I and Phase II metabolism of Phenacetin: Phase 1 (De-demethylation) of phenacetin to paracetamol (acetaminophen), and phase 2 secondary metabolites of acetaminophen via the processes of glucoronidation, sulfonation and N-hydroxylation (B). LC-MS chromatograms: Representative chromatograms of mono- di- and tri-demethylation

of HepaRG cells following challenge with Phenacetin. The parent compound phenacetin is shown at 180 m/z, mono-demethylation shown at 168 m/z, Di-demethylation 152 m/z, and tri-demethylation 138 m/z (C). D: Comparison of mean % turnover of demethylation (comparing mean % of mono- di- and tri-demethylation) between HepaRG and C3A cells.

**Fig. 4 Drug metabolite profiling of testosterone (CYP3A4) using HPLC**

Metabolism of testosterone in HepaRG cells on culture day 8 (A), showed a significant level of the major secondary metabolite 6-Beta-hydroxytestosterone and a wider range of secondary metabolites compared with in C3A cells (B).

**Fig. 5 Flow cytometry profiling of integrin expression in HepaRG and C3A cells**

Flow cytometry histograms comparing integrin expression (CD49a-f:  $\alpha 1$ - $\alpha 6$ ) and CD29 (integrin  $\beta 1$ ) between C3A, HepaRG101 and HepaRG116 cell lines (dark lines); and isotype controls (grey lines). CD49d was not expressed by any of the 3 lines. The expression of CD49a, CD49b and CD29, was comparable between all 3 cell lines, though a proportion of HepaRG116 cells had down-regulated expression of these. Whereas expression of CD49e by HepaRG101 cells was maintained on HepaRG116 cells but absent from C3A cells. This was also true for expression of CD49c, except that there was some down-regulation of expression by HepaRG116 cells. Conversely C3A expressed CD49f, while it was absent or low on HepaRG101 and 116 cells.